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REVIEW ARTICLE

HOW TO CHARACTERIZE A BIOLOGICAL ANTIOXIDANT

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An antioxidant is a substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. Many substances have been suggested to act as antioxidants *in vivo*, but few have been proved to do so. The present review addresses the criteria necessary to evaluate a proposed antioxidant activity. Simple methods for assessing the possibility of physiologically-feasible scavenging of important biological oxidants (superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, haem-associated ferryl species, radicals derived from activated phagocytes, and peroxyl radicals, both lipid-soluble and water-soluble) are presented, and the appropriate control experiments are described. Methods that may be used to gain evidence that a compound actually does function as an antioxidant *in vivo* are discussed. A review of the pro-oxidant and anti-oxidant properties of ascorbic acid that have been reported in the literature leads to the conclusion that this compound acts as an antioxidant *in vivo* under most circumstances.

KEY WORDS: Antioxidant, free radical, lipid peroxidation, ascorbic acid, oxygen radicals, DNA damage, peroxyl radicals, neutrophils, hypochlorous acid.

INTRODUCTION

The word "antioxidant" can be defined in various ways. Often, the term is implicitly restricted to chain-breaking antioxidant inhibitors of lipid peroxidation, such as vitamin E. However, the author prefers a broader definition — an antioxidant is "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate."[1] The term "oxidizable substrate" includes almost everything found in living cells, including proteins, lipids, carbohydrates and DNA. Antioxidants are of interest to radiation chemists, food scientists, polymer chemists and even to curators of museums,[1–4] but I shall confine discussion here to the antioxidants known, or proposed, to be important in aerobic organisms. Recent reviews have covered the well-established physiological antioxidant roles of such proteins as superoxide dismutase,[5] glutathione peroxidase,[6] catalase[1,6,7] and caeruloplasmin.[8] The role of the lipid-soluble chain-breaking antioxidant vitamin E has also been extensively discussed.[9,10]

*The term ROS is used in preference to oxygen radicals (since H_2O_2, singlet $O_2^1\Delta g$ and HOCl are non-radicals) or oxidants (since O_2^- is also a reducing agent). "Reactive" is a relative term, e.g. O_2^- is more reactive than O_2 but much less so than ·OH or HOCl.

TABLE 1
Questions to ask when evaluating the proposed role of an "antioxidant" *in vivo*

1. What biomolecule is the compound supposed to protect? Is the compound present *in vivo* at or near that biomolecule at sufficient concentration?
2. How does it protect — by scavenging ROS, by preventing their formation or by repairing damage?
3. Is antioxidant protection the primary biological role of the molecule or a secondary one? For example, SOD has probably evolved as an antioxidant enzyme.[2] By contrast, transferrin has probably evolved as an iron transport protein, although the binding of iron ions to transferrin stops them accelerating radical reactions,[38] giving this protein an important secondary role in extracellular antioxidant defence.[39]
4. If the antioxidant acts by scavenging a ROS, can the antioxidant-derived radicals themselves do biological damage?
5. Can the antioxidant cause damage in biological systems different from those in which it exerts protection?

Many other substances have been proposed to act as antioxidants *in vivo*. They include β-carotene,[11] albumin,[12] metallothionein,[13] carnosine and related compounds,[14,15] mucus,[16] phytic acid,[17] taurine and its precursors,[18–20] bilirubin,[21] uric acid,[22] oestrogens,[23] creatinine,[24] ergothioneine (reviewed in[25]), dihydrolipoic acid,[26] ovothiols,[27] coenzyme Q,[28] polyamines,[29] retinol,[30] flavonoids and other phenolic compounds of plant origin[31] and ascorbic acid, a compound that has been reported to have both antioxidant and pro-oxidant properties, depending on the reaction conditions.[32,33] Some drugs administered to humans, such as non-steroidal anti-inflammatory drugs (reviewed in[34]), desferrioxamine[35,36] and N-acetylcysteine ([reviewed in[37]]) might have antioxidant properties *in vivo*.

In evaluating the likelihood of a proposal that a given compound acts as an antioxidant *in vivo*, it is important to ask the right questions, as summarized in Table 1. Some quite simple experiments can be performed *in vitro* to answer certain of these questions, and the results can allow one to rule out the proposed antioxidant ability in several cases. The purpose of the present review is to outline a battery of fairly-simple experiments that may be used to approach this problem. Let us first remind ourselves what reactive oxygen species (ROS)* are actually formed *in vivo*.

BIOLOGICALLY-IMPORTANT REACTIVE OXYGEN SPECIES

In testing putative antioxidant activity it is important to use biologically-relevant reactive oxygen species (ROS)*. One of these is superoxide, O_2^-, which is known to be formed *in vivo*.[1,5,40] Some of this O_2^- production is accidental, e.g. by leakage onto O_2 of electrons from the electron-transport chains of mitochondria and endoplasmic reticulum.[1,5,40] Some O_2^- production is deliberate, e.g. that by activated phagocytic cells.[41] It has been suggested that vascular endothelium may also constantly produce small amounts of O_2^- as part of a vaso-regulatory mechanism.[42]

Superoxide formed *in vivo* is converted by superoxide dismutase (SOD) or by non-enzymic dismutation into H_2O_2. Some enzymes, such as glycollate oxidase, also produce H_2O_2 directly.[1,43] Unlike O_2^-, H_2O_2 is able to cross all biological membranes. Both O_2^- and H_2O_2 can find some targets within cells at which they can do direct damage,[5,40] but on the whole their reactivity is limited. Thus only a few compounds, other than specific enzymes such as SOD and catalase, are able to remove O_2^- and

H_2O_2 at rapid rates. For example, many thiols react with H_2O_2 and with O_2^-, but the rate constants for these reactions are low, usually $< 10^3 M^{-1} s^{-1}$.[37,44] Thus very high thiol concentrations (often > 1 mM) would be required to achieve significant scavenging. It is therefore unlikely that most thiol compounds administered to humans as drugs (such as N-acetylcysteine or penicillamine) could act *in vivo* by scavenging O_2^- or H_2O_2, simply because such high drug concentrations are not achieved in body fluids. GSH is present at intracellular concentrations in the high millimolar range, but the reaction of GSH with O_2^- or H_2O_2 can produce reactive sulphur-containing radicals that might be capable of doing more damage than the O_2^- and H_2O_2 would by themselves.[45,46] It is perhaps therefore fortunate that reactions of O_2^- and H_2O_2 with thiols are slow.

Ascorbic acid reacts with O_2^- with a rate constant of approximately $2.7 \times 10^5 M^{-1} s^{-1}$ at pH 7.4.[47] Many animal tissues (e.g. eye lens, lung, brain) and the chloroplasts of green plants contain ascorbate at millimolar concentrations, and so scavenging of O_2^- *in vivo* might be feasible.[47-49] Thus although SOD reacts with O_2^- about four orders of magnitude faster than does ascorbate, the concentration of ascorbate *in vivo* is often more than four orders of magnitude greater than that of SOD.[1,48,49] Such arguments based on rate laws assume, of course, completely homogeneous distribution of substances within the cell, which is rarely the case. For example, most O_2^- formed in chloroplasts is produced by the thylakoids. Much SOD is bound to the thylakoid surface, whereas ascorbic acid is in the stroma, giving the SOD an advantage.[1,48] Reaction of ascorbic acid with O_2^- produces the semidehydroascorbate radical, which appears to be an unreactive species, incapable of doing significant biological damage.[50,51] Indeed, the stability of this radical means that it can easily be observed by ESR in biological material.[50]

Several keto-acids, such as glyoxylate, pyruvate and 2-oxoglutarate[52-56] react with H_2O_2. Rate-constants for the reactions have not been determined but are likely to be low, since millimolar concentrations of keto-acid have to be added to achieve high rates of H_2O_2 removal[53-56] and it is not clear if such concentrations are usually achieved *in vivo*. Pyruvate at high concentrations in cell-culture media can protect cells against damage by H_2O_2 [53,56] and Varma *et al.*[55] have argued that pyruvate concentrations in the mammalian lens might be sufficiently high to offer protection against H_2O_2 *in vivo*.

Hydroxyl radical

Much of the damage done by O_2^- and H_2O_2 *in vivo* is thought to be due to their conversion into highly-reactive oxidants.[1,5,35,39,57,58] After a long debate, it seems to be firmly established that one of these oxidants is hydroxyl radical, $\cdot OH$,[57-59] confirming a view held by the author[60] and others[61-63] for over ten years now.

Formation of $\cdot OH$ from O_2^- requires traces of catalytic transition metal ions, of which iron seems likely to be the most important *in vivo*[57,64] although copper ions might also play a role.[58,65] In systems containing O_2^-, H_2O_2 and iron ions, reactive species additional to $\cdot OH$ are probably also formed, including perferryl and ferryl (Figure 1). With copper ion/H_2O_2 systems the chemistry is even less clear — debate continues as to whether $\cdot OH$ is formed at all, or if it is formed *in addition to* a reactive copper(III) species (e.g. $Cu(OH)_2^+$).[58,65,66] However, the ability of copper ion/H_2O_2 systems to do severe damage to proteins[65,67-69] and to DNA[70,71] is well established, so that whatever species are formed are highly reactive. Even in systems containing iron